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GROWTH OF IRIS EPITHELIAL CELLS OF *NOTOPHTHALMUS VIRIDESCENS IN VITRO*: ANALYSIS OF THE CELL CYCLE

by

© LOUISE PATRICIA HORSTMAN

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Growth of Iris Epithelial Cells of Notophthalmus viridescens in Vitro: Analysis of the Cell Cycle," submitted by Louise P. Horstman in partial fulfilment of the requirements for Master of Science.

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ABSTRACT

The objective of this work was the development of an *in vitro* system for studying cellular metaplasia occurring during lens regeneration from the dorsal iris of the newt. Epithelial cells from the normal iris and the ten-day regenerating iris were used. The behavior and growth of these cells in culture are described.

Depigmentation begins prior to mitosis and concurrent with cell attachment to the substrate. A small non-dividing population of heavily pigmented cells remains in the normal and ten-day iris cell cultures. The lag phase is considerably longer in normal than in ten-day iris cell cultures. A doubling time of 85 hours was found in the ten-day iris cell cultures and 150 hours in the normal iris cell cultures. Exponential growth is due to proliferation of fully depigmented cells. Repigmentation occurs during the stationary growth phase.

The cell cycle was measured by autoradiography. In both cultures the DNA synthetic period is 36 hours and G_2 6 hours. In the ten-day iris cell cultures the total cell cycle time is 69 hours and G_1 25 hours. An estimate of the total cycle time in the normal iris cell culture was made using the synthetic index. It is concluded that at least in regard to cell cycle parameters the *in vitro* system does not differ greatly from that observed *in vivo*.

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INTRODUCTION

A. Cellular Metaplasia

Cellular differentiation in multicellular organisms is the biosynthetic specialization of a cell reflected in its particular organization and function. In vivo, the characteristics of a fully differentiated cell are usually stable and transferred to daughter cells upon cell division. However under certain conditions, such as during restoration of tissue, these characteristics may be shed as cell proliferation occurs. Upon redifferentiation the original cell type is usually acquired by daughter cells. In some instances, however, the daughter cells are of another type. The latter phenomenon has been referred to as cellular transformation or cellular metaplasia. Since cell differentiation is a result of the selective expression of genetic information, metaplasia must involve a change in some mechanism controlling this expression.

While an understanding of metaplasia is important to developmental biology, its occurrence has not been demonstrated unequivocally in the majority of cases reported. The occurrence of metaplasia during the replacement of differentiated eye tissue in embryos throughout the vertebrate series has been reported. Examples have been cited in anurans (Ikeda, 1938; Overton and Freeman, 1960), urodeles (Sato, 1930; Stone, 1943; Reyer, 1948; and others), teleosts (Dabaghian, 1959; Sato, 1961), birds (Maisel and Langman, 1961; Coulombre and Coulombre, 1965) and mammals (Stewart and 'Espinasse, 1959). The capacity for metaplasia in these cases is apparently lost long before adulthood, with the exception of two loach species (Sato, 1961) and thirteen species of the family Salamandridae. In the adult newts remarkable regenerative abilities exist throughout the body. In the eye, a lens

can regenerate from the dorsal iris; and the neural retina, iris, and optic nerve can regenerate from the pigmented layer of the retina (Wachs, 1920; Stone, 1950; Hasegawa, 1958). Lens regeneration has been the most thoroughly studied of these phenomena. This is partly due to the ease of lentectomy and partly to the demonstration that the cells involved in lens regeneration arise from a single tissue source (Yamada, 1967).

B. History

Lens regeneration from the iris of adult newts was first described by Colucci in 1885. A series of studies by Wolff (1894, 1895, 1901, 1904) resulted in the term Wolffian lens regeneration. Since then numerous surgical manipulations of the eye have led to the following conclusions about lens regeneration from the iris.

The eye chamber contains factors required for regeneration. An iris grafted elsewhere in the body will not form a lens unless it has been grafted after a certain stage of lens formation has been attained (Ikada, 1936). However, if accompanied by neural retinal tissue, an iris grafted to another part of the body can form an incomplete lens (Stone, 1958a). The eye chamber of non-regenerating species of newts can support lens formation by the iris of regenerating species; the reciprocal arrangement does not result in lens formation (Ikeda, 1934; Amano and Sato, 1940; Amatatu and Fujita, 1941; Reyer, 1956). The position of the iris within the eyechamber determines the size and epithelial-to-fiber ratio of the resulting lens (Sato, 1930).

All areas of the iris are not equally capable of forming a lens when explanted to a host eyechamber. The ability to regenerate a lens declines with distance

from the middorsal pupillary region, and the ventral iris is not able to form a lens at all(Sato, 1930; Mikami, 1941). However, the ciliary border of the dorsal iris can form a lens when the dorsal iris is rotated 180 prior to lentectomy (Stone, 1954a). Several lenses can form simultaneously at the edges of incisions in the dorsal iris provided the edges are kept open (Stone, 1954b).

The neural retina must be present in the eye for lens regeneration to take place. However, a regenerating neural retina, not yet fully differentiated, will support lens formation by the iris (Stone, 1950; Stone and Steinitz, 1953; Hasegawa, 1958; Reyer, 1971). An artificial barrier preventing molecular exchange between iris and neural retinal tissue prevents lens regeneration (Stone, 1958b).

Reinsertion of the original lens following lentectomy, contrary to the insertion of other types of tissue, prevents lens regeneration (Fischel, 1902). Contact between lens and iris may be critical for the stimulation of regeneration, since a lens that fails to touch the iris upon insertion allows regeneration to begin, and an inserted lens smaller than the original lens will prevent regeneration if touching the iris (Wachs, 1914). A product of lens metabolism may be responsible for inhibiting differentiation of lens tissue. Irises implanted into the vitreous chamber with the host lens present will regenerate lenses of smaller size and with less frequency than if the host lens is absent (Frost, 1961). Smith (1965) isolated a fraction of lens extract which inhibits regeneration in the lentectomized eye.

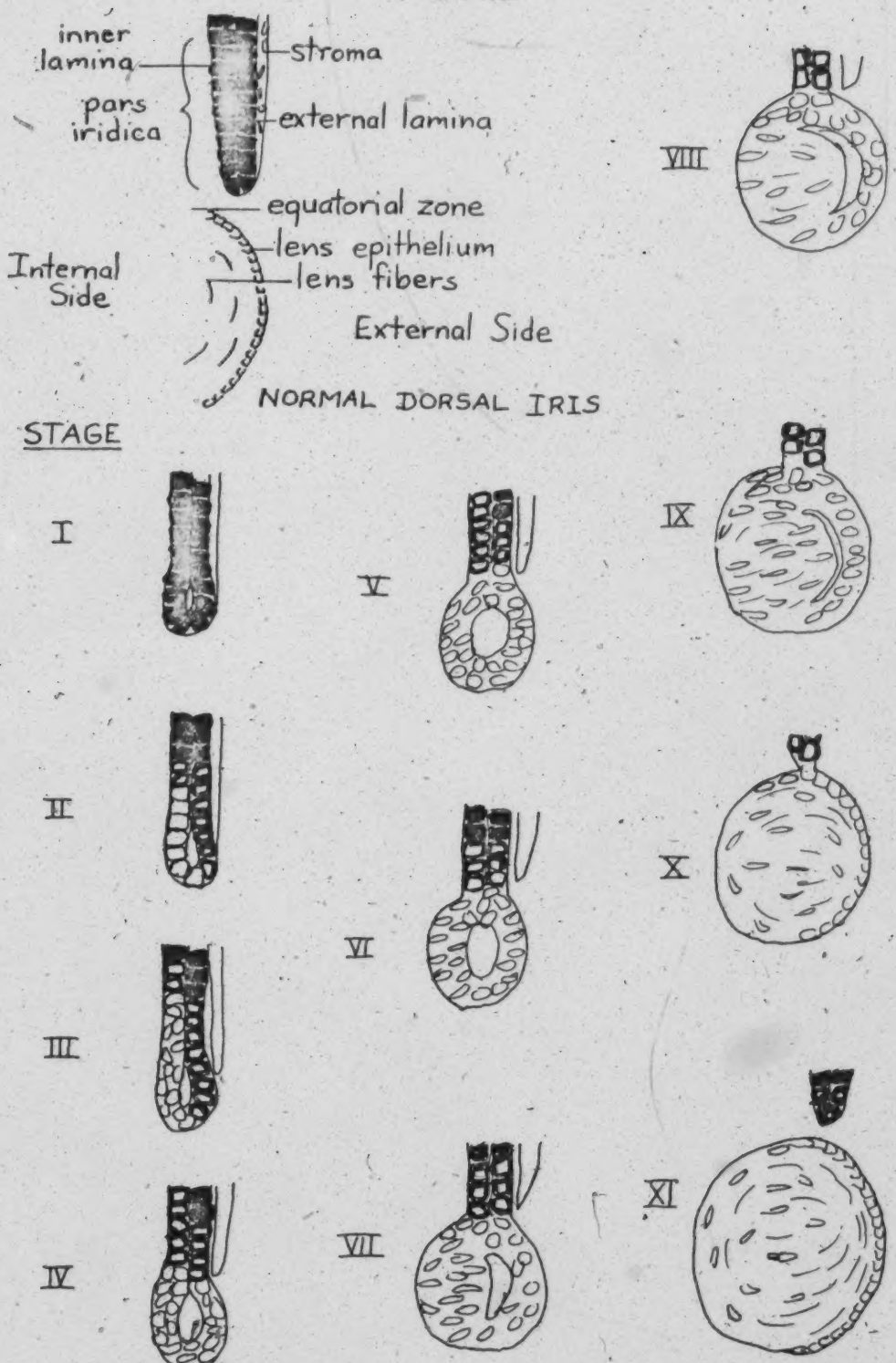
C. Events in Lens Regeneration

The events occurring during lens regeneration have

been assigned to specific stages in the European newt Triturus taeniatus and its Japanese counterpart Triturus pyrrhogaster (Sato, 1930). The following description of lens regeneration in the eastern newt Notophthalmus (Triturus) viridescens has been taken from Yamada (1967). Diagrams corresponding to the stages described appear in Figure 1.

Stage	Day	Events
normal	0	The iris is an epithelium covered by a layer of connective tissue. In the dorsal iris, the epithelium consists of two layers, the external (corneal) lamina and internal (retinal) lamina, which join at the pupillary border. The cells of the epithelial layers contain melanosomes and can be referred to as melanocytes (Fitzpatrick et.al., 1966). The pupillary half of the epithelium, or pars iridica, is more heavily pigmented than the retinal half, or pars ciliaris. The connective tissue layer, or stroma, is vascularized and contains iridophores as well as connective tissue cells.
I	3-6	The space between inner and external layers of the dorsal iris becomes apparent. Epithelial cell nucleoli appear.
II	6-10	Melanosome extrusion begins by cells at the middorsal pupillary border. Phagocytic cells appear in the vicinity of depigmenting cells.
III	8-11	Depigmentation continues. Cells of the internal lamina are almost completely depigmented. Ribosomal content increases in depigmenting cells.
IV	9-15	The separated layers begin to form a vesicle, composed mainly of depigmented cells, at the middorsal pupillary border.
V	12-15	The vesicle enlarges by cell multiplication and addition of depigmented cells. The inner layer of the vesicle thickens. All cells show increased cytoplasmic basophilia.
VI	12-16	Cells in the inner layer of the vesicle elongate, stop dividing, lose basophilia, and begin synthesizing lens proteins. A stalk forms by constriction of the layers

FIGURE 1


inner lamina
stroma
pars iridica
external lamina
equatorial zone
lens epithelium
lens fibers
Internal Side
External Side
NORMAL DORSAL IRIS
STAGE
I
II
III
IV
V
VI
VII
VIII
IX
X
XI

		between iris and vesicle. Cell proliferation in the stalk continues.
VII	15-18	The inner layer protrudes into the vesicle cavity due to continued elongation of inner cells, which are starting to differentiate into primary lens fibers.
VIII	15-19	Vesicle cavity is almost filled by primary lens fiber cells. Prospective secondary fiber cells, located between the primary fiber cells and the cuboidal external cells of the lens vesicle, begin to elongate.
IX	18-20	Cells of the external layer of the vesicle flatten, continue to divide, and surround the fiber cell mass. Elongation and differentiation are occurring in the secondary fiber cells. The latter are joined by additional elongating cells at the equatorial region of the lens vesicle.
X	18-25	Secondary fiber cells surround the core of primary fibers. The lens epithelium thins, becoming a single cell layer.
XI	21-28	Secondary fiber cells grow. Lens epithelial cells continue to divide. The stalk disappears, separating the lens from the iris.

Demonstration of the occurrence of metaplasia during lens regeneration requires the identification of another differentiated tissue as the source of the lens cell population. Autoradiographic studies of the incorporation of ^{3}H-thymidine into DNA in the iris at progressive stages of regeneration have traced the cell lineage of the developing lens. Yamada and Roesel (1969, 1971) have shown that the iris epithelium is a static tissue in which no uptake of ^{3}H-thymidine can be detected prior to lentectomy, and to which no cells are contributed by germinative zones of other tissues. By 3 to 4 days after lentectomy the first label is found in the iris, at the dorsal and ventral pupillary borders (Eisenberg and Yamada, 1966; Yamada and Roesel, 1969; Reyer, 1971). The percentage of labelled cells increases

up to day 5, when mitosis begins. Autoradiographs made at various intervals after injection of ^{3}H-thymidine show the spread of labelled cells from the dorsal pupillary border of the iris into all areas of the developing lens vesicle (Eisenberg and Yamada, 1966; Reyer, 1971). In another approach to the demonstration of metaplasia, Zalik and Scott (1971) showed that a stage III pigmented iris labelled with ^{3}H-thymidine will give rise to a labelled lens when transplanted to the optic chamber of a host newt.

On the basis of combined autoradiographic and immunofluorescent studies (Takata et.al., 1964, 1966; Yamada and Takata, 1965), three subpopulations can be distinguished in the developing lens vesicle: the prospective primary lens fiber cells, which will constitute the lens fiber core at the internal side of the vesicle and are the first to cease DNA synthesis, elongate, and synthesize the lens-specific alpha, beta, and gamma crystallins; the prospective secondary lens fiber cells, which undergo the same sequence of events after further cell replication; and the lens epithelial cells, located on the external side, which constitute the proliferative zone of the lens vesicle and synthesize alpha and beta crystallins while continuing to divide. In the equatorial zone of the lens the latter cells eventually differentiate into lens fibers.

In the transforming iris melanocyte, the following sequence of events take place, as deduced from numerous studies: a change in nucleolar ultrastructure and number (Eguchi, 1964; Karasaki, 1964; Dumont et.al., 1970); enhanced incorporation of labelled uridine into ribosomal RNA (Reese et.al., 1969); rounding of the nucleus and increase in nuclear volume (Eguchi, 1963; Dumont et.al., 1970); increased protein synthesis (Yamada and Takata, 1963); onset of DNA synthesis (Eisenberg and Yamada,

1966); and mitosis. Mitosis is first seen in the iris 4 days after lentectomy (Yamada and Roesel, 1971). Depigmentation begins prior to mitosis in some cells and after mitosis in others (Yamada, 1967). Cells in the proliferating zone of the developing vesicle of N. viridescens show a steady decrease in mean generation time as regeneration proceeds, from approximately 200 hours at day 5 after lentectomy to about 50 hours at day 30. In cells of the 15-day regenerating lens vesicle, which have a generation time of about 65 hours, the length of the DNA synthetic period was estimated to range between a minimum value of 19 and a maximum of 33 hours. The G_2 phase was estimated to be 2 hours; G_1, 40 hours; and mitosis, 1.3 hours (Zalik and Yamada, 1967).

The process of depigmentation may have a determining role in the transformation of the iris cell. Eguchi (1963) and Karasaki (1964) described the shedding of melanosomes in membrane-bound vesicles formed at the cell surface. Electrophoretic mobility studies have revealed a significant decrease in surface charge density in the depigmenting cells of the activated iris. This is evident in the cells from 10- and 15-day regenerates and is due at least in part to a loss of ribonuclease- and neuraminidase-sensitive groups from the cell surface (Zalik and Scott, 1972). After completion of depigmentation only a thin layer of cytoplasm surrounds the nucleus. Yamada (1972) has suggested that loss of specialized cell products accompanied by alteration of the cell surface may allow new factors to enter the cell and influence the genome, resulting in redifferentiation into a different cell type.

D. Studies in vitro

In order to define the factors in the eye which

are responsible for lens regeneration, a number of investigators have resorted to organ culture methods. When the isolated iris is cultured under conditions which do not allow cell migration, enhanced incorporation of ^{3}H-uridine into rRNA, incorporation of ^{3}H-thymidine into DNA, and increase in nucleolar number is observed (Reese, 1971). Depigmentation in these culture conditions requires the presence of another tissue, which need not be from the eye, such as muscle or lung (Connelly et.al., in press). Depigmentation will continue in cultured iris explants after having previously begun *in vivo* (Zalik and Scott, 1969). Cell proliferation in iris explants cultured on agar will take place only if cell division had already begun prior to explantation (stages IV-V); likewise, fiber differentiation will occur only if it had already started *in vivo* (stages VI-VII). Thus there appear to be three phases requiring external factors for their initiation: depigmentation, proliferation, and redifferentiation (Zalik and Scott, 1969). Total lens regeneration has been obtained from the dorsal iris cultured together with frog neural retina (Yamada and McCevitt, 1971) and from the dorsal iris cultured with cornea and newt pituitary (Connelly et.al., in press).

E. Purpose of Thesis

A cell culture system would enable further study of the factors involved in cellular metaplasia. The advantages of controlled culture conditions are obvious. The processes involved in depigmentation, proliferation, and redifferentiation and the role of external factors on these processes could be studied in a defined environment. Clones of iris epithelial cells giving rise to lens cells would provide the ultimate demonstration of metaplasia. This thesis is the first attempt to describe

the growth and behavior of the iris epithelial cells of N. viridescens under cell culture conditions. The pattern of culture growth, cell pigmentation and proliferation is described in cell cultures from the normal and de-differentiating iris. In addition, studies on the cell cycle and the duration of its component phases are presented.

MATERIALS AND METHODS

Adult newts of the species Notophthalmus viridescens, obtained from Donaldson, Tennessee, and kept in dechlorinated water at 12° C., were used for all work described in this thesis.

A. Lentectomy

The method of Eisenberg and Yamada (1966) was employed for lentectomizing newts. Animals were anaesthetized in 0.1% tricaine methanesulfonate (MS-222; Sandoz). Using a sharp blade, a horizontal incision was made in the cornea directly over the pupil. The lens was forced through this incision by application of gentle pressure with forceps on the upper and lower edges of the cornea. Care was taken to avoid damage to the iris. Lentectomized newts were kept in dechlorinated water at 24° C. for ten days, at which time their irises were removed as described below.

B. Dissociation of Cells for Culture

The same procedure was used to obtain iris tissue from both normal and previously lentectomized newts. Approximately 30 irises were used per experiment. Animals were immersed in dilute potassium permanganate solution for one hour to sterilize their skin and anaesthetized in 0.1% MS-222 containing 100 U/ml Penicillin and 100 µg/ml Streptomycin. They were immersed briefly in 70% ethanol and the eyes rinsed with a stream of sterile distilled water. Dorsal irises were excised by the method of Zalik and Scott (1969): a corneal incision over the pupil was made, or reopened in the case of lentectomized animals, and extended to the medial and lateral edges of the iris with the help of iris scissors. The incision was completed around the upper margin of

the iris and the dorsal irises with overlying corneas were placed in amphibian Ringer's solution of the following composition:

NaCl	6.50 g
KCl	0.14 g
trizma buffer (tris(hydroxymethyl)aminomethane and hydrochloride) pH 7.8, 0.004 M	0.61 g
glucose	1.80 g
$MgSO_4$	0.10 g
$CaCl_2$	0.12 g
distilled H_2O	1 liter
Gentamycin (Schering)	50 µg/ml

Irises were then dissected free of corneal and other tissue and washed twice in Ca, Mg-free amphibian Ringer's solution (CMFR) of the same composition as above with $MgSO_4$ and $CaCl_2$ omitted. One of two alternative methods of cell dissociation was then used. In the enzymatic method, irises were incubated for 5-8 hours at 4°C. on a rotory shaker in a medium modified from Seto and Rounds (1968):

NaCl	6.80 g
KCl	0.10 g
Na_2HPO_4	0.11 g
$NaHCO_3$	0.20 g
glucose	1.00 g
trypsin (Difco 1:250)	2.50 g
fetal calf serum (Grand Island Biological Co.)	25 ml
distilled H_2O	1 liter
Gentamycin	50 µg/ml
pH adjusted to 7.4	

Following incubation the cells were separated by gentle pipetting for approximately 3 minutes, collected by centrifugation at 400 x g for 7 minutes and washed twice

by centrifugation at 250 x g in Ringer's solution. In the alternative method, irises were incubated in CMFR for 5-8 hours at 24°C. This treatment loosens the iris pigment cells from the more adherent cells of the iris stroma. Gentle pipetting for 15-20 minutes was then necessary for complete separation of cells. The cell suspension was pipetted through a stainless steel screen to remove stromal tissue fragments and cells were collected by centrifugation at 400 x g for 10 minutes. All glassware and screens used for cell dissociation were siliconized prior to each experiment. Cell yield was determined by hemocytometer count and an estimation of viability was made using extrusion of trypan blue within 10 minutes as a criterion for viability. The viability test was possible only with depigmented or partially depigmented cells from the ten-day irises.

In preliminary studies the trypsin method of dissociation was found to yield a much higher proportion of non-melanocytes in culture than the CMFR method. For this reason the CMFR method was used for all further studies. The early behavior in culture of cells obtained by the two methods is compared in Results.

Although dissociation of cells in CMFR appears to remove preferentially the iris melanocytes, it does not yield a pure melanocyte population. From observation and cell counts in the hemocytometer, melanocytes were found to comprise approximately 50% of the total cell yield; another 25% consisted of red blood cells, and the remaining 25% were assorted non-melanocytes of various size. The latter could be derived from the connective tissue and blood vessels in the iris stroma, and thus could include endothelial cells, fibroblasts from the connective tissue, iridophores, white blood cells, and macrophages. The behavior of these cells in culture is

described in Results.

C. Cell Culture

Iris cells were grown in plastic tissue culture dishes (Falcon Plastics) containing 5 mls of modified Leibovitz medium (Leibovitz, 1963), which consisted of L-15 Leibovitz medium (Gibco) diluted 50:50 with distilled water and supplemented with 10% fetal calf serum (Gibco) and 50 µg/ml Gentamycin. Approximately 2×10^4 cells were seeded per dish. Cultures were fed weekly and maintained in a humid atmosphere at 24° C. Cells which did not remain attached to the substrate, including all red blood cells, some melanocytes, and some nonpigmented cells, were eliminated after a few changes of medium.

Upon formation of monolayers, primary cultures were subcultured by the method of Freed and Mezger-Freed (1970). In order to detach cells from the substrate, plates were treated with a medium consisting of:

NaCl	2.75 g
KCl	0.27 g
Na_2HPO_4	0.77 g
glucose	0.07 g
bovine albumin powder (Fraction V, Sigma Chemicals)	1.50 g
trypsin (Difco, 1:250)	5.00 g
distilled H_2O	1 liter

pH adjusted to 7.4 with 0.5 M NaOH.

Culture dishes were washed three times with the above medium lacking the trypsin component; they were then incubated in the same medium containing trypsin at 24° C. until the majority of cells had detached (about 20 minutes). Cells were then washed three times by centrifugation at 250 x g in Ringer's solution and cell yield was determined by hemocytometer count. For estimation of

cell population growth in primary subcultures, approximately 2 x 10^4 cells were seeded per dish and the increase in cell number determined as described below.

To determine whether macrophages and other white blood cells were present as part of the attached cell population in cultures, attempts were made to establish cultures of these cells under the same conditions as those for iris cells. Newts were injected intraperitoneally with 0.05 g oyster glycogen (Fisher) in 4 mls of Ringer's solution. The following day the peritoneal cavity was flushed with additional Ringer's, the peritoneal exudate withdrawn by syringe, and cells collected by centrifugation. These were plated and cultured in conditioned medium which had been removed from growing iris cell cultures, filtered, and supplemented with 15% fetal calf serum. The conditioned medium was changed once a week. A variety of cell types were present in these cultures and several types attached and spread on the substrate. However, after 10 days in culture cells began to detach, and by two weeks all had detached. Reattachment did not occur during the following two weeks. Similar results were obtained for several attempts. Decreasing the serum concentration to 10 and 5% gave the same results.

D. Estimation of Cell Population Growth

To measure the growth rates of cell cultures obtained from normal irises (normal cultures) and ten-day regenerating irises (ten-day cultures), the increase in cell number at successive time intervals in culture was measured. Cell counts were made using an inverted phase microscope equipped with an eyepiece calibrated with rectangular fields. Magnification and field were selected to include a minimum of 10 and maximum of 100 cells

per field. A minimum of 400 cells were counted for each plate, giving a 95% confidence interval of approximately 20% of the estimated number of cells (Freed and Mezger-Freed, 1970). From the ratio of the area of the rectangular field to the area of the culture dish the number of cells per dish was calculated. Since cell density tended to be higher in the center of a plate, one entire plate or, in the case of high density cultures, one half a plate, was counted by scanning its surface in equidistant parallel lines, making counts at equally spaced intervals along each line. Only attached cells with nuclear area lying more than halfway within the field were counted. Multinucleate cells were counted as one cell.

Pigmentation was scored using phase contrast optics. At least 400 cells were scored per plate and plates were scanned in the same manner as for cell counting. Each cell was assigned to one of three categories: heavy, medium, and light-to-no pigmentation. Only well-spread cells were scored, since cells appear more heavily pigmented when rounded up.

E. Estimation of Cell Population Kinetics

The cell cycle and other cell population parameters were studied in normal and ten-day cultures using autoradiography. The pulse-chase method of Quastler and Sherman (1959) was used to measure the duration of the cell cycle and its component phases. For autoradiography, monolayers of primary cultures were subcultured as above onto Corning #1 8 x 50 mm coverslips. Experiments were carried out during the logarithmic phase of growth.

A preliminary experiment was done to find the shortest time of incubation in ^{3}H-thymidine that would give an adequate grain count. According to Cleaver and Holford (1965), a concentration of 3×10^{-6} M thymidine

gives maximum rate of incorporation into DNA by cells in culture. To obtain this concentration, ^{3}H-thymidine of specific activity 6.7 Ci/mmole (New England Nuclear) was diluted 1:40 with unlabelled thymidine to give a final concentration of 0.5 μCi/ml of medium. Coverslips supporting growing cells were transferred to the medium supplemented with 0.5 μCi/ml and incubated for periods ranging from 15 minutes to 2 hours. After the desired incubation time coverslips were returned to the original cultures dishes for 9 hours, rinsed in Ringer's, fixed in methanol for 1 minute, mounted on slides, and dried. Slides with attached coverslips were then dipped in NTB-2 liquid emulsion (Eastman Kodak) and exposed at room temperature for 15 days. They were developed in Kodak D-11 for 2 minutes, placed in Kodak Fixer for 4 minutes and stained with Ehrlich hematoxylin. It was found that 30 minutes was the shortest incubation time that would yield a grain count sufficiently above the background level to facilitate rapid identification of labelled cells.

Many investigators (Sisken, 1964; Cleaver, 1967; Bischoff and Holtzer, 1969) have found it necessary to incubate cells previously exposed to ^{3}H-thymidine in medium containing unlabelled thymidine at about 100 times the concentration of the labelled nucleoside in order to rid the intracellular pool of acid-soluble ^{3}H-thymidine. To determine whether such a concentration of thymidine would inhibit culture growth as reported by many investigators (Hakala and Taylor, 1959; Morris and Fischer, 1960; Xeros, 1962; and others), preliminary experiments were conducted in which 3.2×10^{-4} M thymidine was added to the culture medium of two replicate subcultures and cell counts made daily for 3 days.

For each measurement of the cell cycle, approximately

1.5×10^5 cells were subcultured onto coverslips placed in a 150 x 20 mm culture dish containing 25 mls of medium. During logarithmic culture growth the coverslips were transferred to plates containing fresh medium with ^{3}H-thymidine (0.5 μCi/ml). After 30 minutes in label the coverslips were rinsed in medium containing 3.2×10^{-4} M unlabelled thymidine and returned to the original culture dish with 3.2×10^{-4} M thymidine added to the original medium. After this treatment coverslips were removed at each of the following intervals in culture: 4, 8, 12, 16, 22, 28, 34, 42, 51, 60 and 70 hours for the normal culture and 4, 8, 10, 13, 17, 22, 28, 37, 50, 58, 74, 84, 96 and 108 hours for the ten-day culture. Coverslips were rinsed in Ringer's solution, fixed in methanol and mounted on slides. Autoradiographs were processed as described previously.

RESULTS

A. Results of Dissociation of Iris Tissue

Total yield following dissociation of irises in Ca, Mg-free Ringer's solution was approximately 700 melanocytes per dorsal iris, while dissociation with trypsin gave a total yield of about 1500 melanocytes. About 80% viability was indicated by dye exclusion of depigmented cells from ten-day regenerating irises dissociated in CMFR.

B. Behavior of Iris Cells in Culture

Normal Cultures

The majority of melanocytes obtained by dissociation in CMFR require at least 2 days after plating to become attached to the substrate, with some cells requiring much longer. One of the first signs of depigmentation is the loss of pigment granules from the central region of the cell. This occurs as the cell membrane attaches to and spreads over the substrate, and as a consequence pigment cell nuclei previously covered with melanin granules become visible. Concurrent with cell attachment and membrane spreading, shedding of pigment granules at the cell periphery occurs. By 2 days a small number of melanocytes are fully attached and depigmentation over the nucleus is complete. By 7 to 8 days about half the population is attached with depigmentation underway. Nuclei of depigmenting melanocytes are kidney-shaped when first visible. Nucleoli and condensed chromatin are visible as soon as the nucleus is uncovered, and are of variable size and number. By the onset of mitosis 5 to 6 large round nucleoli are present. In older cultures this number is reduced to 3 or 4. The ratio of nuclear-to-cytoplasmic volume appears to increase greatly in depigmenting cells within the first week. The nucleus

becomes round or ellipsoid. Blebs or clear bubbles are sometimes observed protruding from partially depigmented cells. Some cells appear to have migrated and left behind on the substrate a trail of cytoplasm.

During the depigmentation phase, macrophages are active in the cultures, moving over the surface of attached and unattached melanocytes. Other nonpigmented cells, presumably white blood cells, are also present in suspension. By one week the majority of red blood cells have detached from the substrate and only a small minority of the attached cell population are non-melanocytes. This consists of iridophores and round, transparent cells, the latter being about one-third the size of melanocytes and having no spread membranes. No attached fibroblasts are present.

Mitosis begins at about 21 days and is found to occur earliest in melanocytes that appear to be in direct contact with each other. The enzymatic method of dissociation accelerates the timing of these early events in culture: in cultures of trypsinized cells a larger proportion of melanocytes are fully attached after 3 days, and mitosis is first seen at 15 days.

The lag phase of growth, or interval between mitosis and exponential growth, occupies about 3 weeks in normal CMFR-dissociated cultures (see Figure 2). During early logarithmic phase growth, the cells assume a fibroblast-like spindle shape, and are often aligned in the same direction within an area. By late log phase, epithelial sheets of cells form, and the cells take on a cuboidal shape (Figure 3). At this stage numerous multilayered clusters of cells appear over the entire culture dish (Figure 4); whether these result from a local rolling up of epithelial sheets or from active cell migration and aggregation is not clear.

FIGURE 2: Growth curve of primary normal culture. A minimum of 400 cells was counted for each point. Lines from each point represent 95% confidence limits. (See Text).

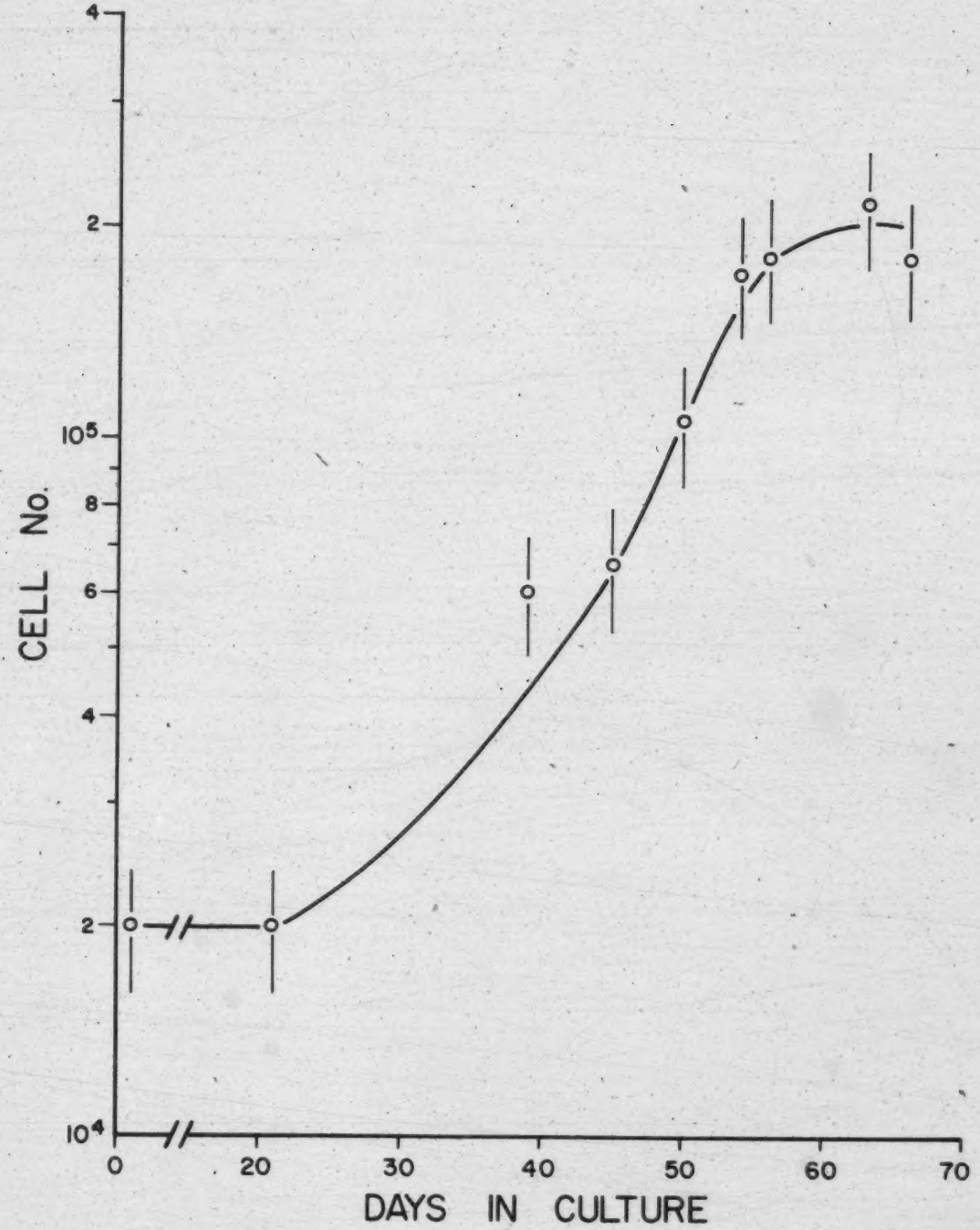
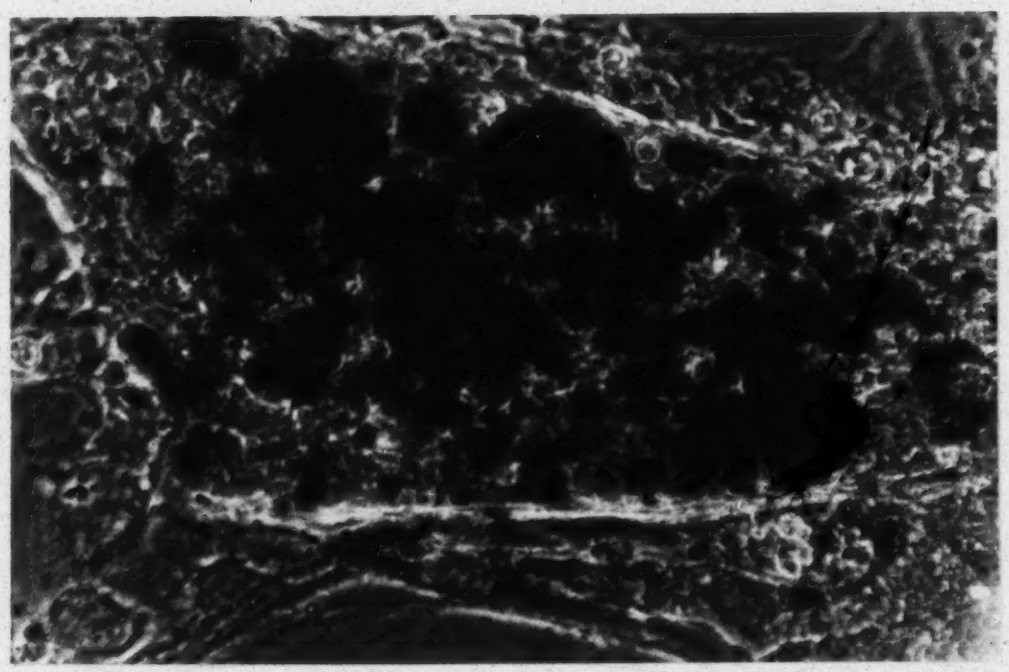

CELL No.
4
2
10⁵
8
6
4
2
10⁴
0
20
30
40
50
60
70
DAYS IN CULTURE

FIGURE 3: Primary normal culture at the end of the logarithmic phase. Most of the cells have acquired a cuboidal shape. 3-6 nucleoli are evident. Magnification X 340.

FIGURE 4: Cell cluster in a primary normal culture. Cells are repigmenting. Magnification X 340.



Exponential growth lasts for about 10 days and is followed by a stationary phase. During this phase proliferation takes place primarily at the boundaries of the forming monolayer and heavily granulated, presumably necrotic cells are seen in the center of the culture dish. Refractile structures are sometimes present in the cell clusters.

The time required for the number of cells in a culture to double can be calculated from the slope of the growth curve according to the equation

$$\text{doubling time} = \frac{t_2 - t_1}{\log_2 x_2 - \log_2 x_1},$$

t_1 and t_2 representing times at which cells were counted and x_1 and x_2 the number of cells at these times (Freed and Mezger-Freed, 1970). The slope of the exponential part of the growth curve can be determined by linear regression. When this is done for the curve in Figure 2 a doubling time of 150 hours is obtained.

The cytoplasm adjacent to the nucleus is the last area to lose pigment granules, and some of the melanocytes retain pigment granules in this area. The proportion of heavily, moderately, and lightly or nonpigmented cells occurring in the culture represented in Figure 2 is shown in Figure 5A. As can be seen from this figure, the heavily pigmented cell population shows no increase throughout the logarithmic phase of culture growth. The moderately pigmented population shows a slow arithmetic increase during this phase, while the lightly and nonpigmented population shows exponential increase. During the stationary phase the number of heavily pigmented cells can be observed to increase greatly (not shown).

Ten-day Cultures

The sequence of events in ten-day cultures is the same as that of normal cultures, but the timing of the

events is accelerated. By 3 days many melanocytes are well-spread, and mitosis is first seen at day 12. The lag phase occupies about 12 days, followed by an exponential phase of about 10 days. Figure 6 is the growth curve for two replicate ten-day cultures. The mean doubling time during the exponential phase is approximately 85 hours. The growth rate declines when the cultures reach 1.5 to 2.0 x 10^5 cells. Cell number continues to increase slowly after this, reaching a density of 3.8 x 10^5 cells at 85 days.

Dissociation with trypsin results in mitosis as early as day 9 in some ten-day cultures.

Figures 5B and 5 C show the change in pigmentation of the two ten-day cultures represented in Figure 6. The pattern is similar to that seen in Figure 5A. The heavily pigmented cell population does not increase in number during the log phase of culture growth. During the stationary phase, however, this population increases dramatically. The moderately pigmented population undergoes an arithmetic increase during log phase followed by a levelling off during the stationary phase. Lightly and nonpigmented cells increase logarithmically throughout the log and stationary phases.

Primary Subcultures

Subcultures from normal and ten-day monolayer cultures show little or no lag phase before exponential growth, and have a doubling time similar to the primary cultures from which they were obtained. Figure 7 is the growth curve for four replicate subcultures from the normal culture of Figure 2. The mean doubling time for the subcultures in Figure 7 is 148 hours.

Heavy repigmentation is observed as subcultures reach confluency (Figure 8).

FIGURE 5: Growth of heavily, moderately, and lightly pigmented populations. A represents a primary normal culture; B and C represent two sister primary ten-day cultures. A minimum of 400 cells was scored at each time interval.

Open circles = lightly or non-pigmented cells; closed circles = moderately pigmented cells; open triangles = heavily pigmented cells.

lag = lag phase of culture growth

log = logarithmic phase of culture growth

stat = stationary phase of culture growth

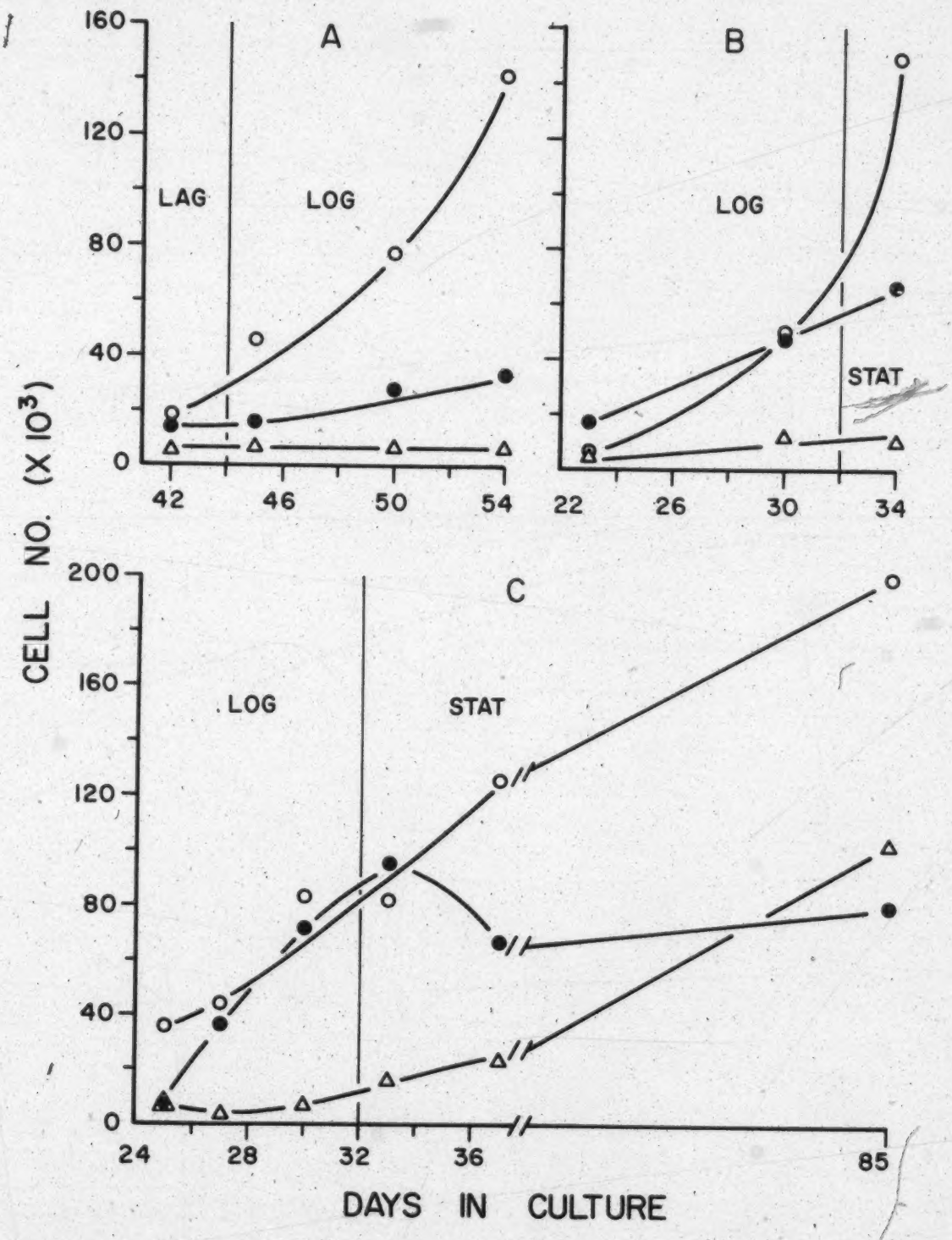

A
B
C
LAG
LOG
LOG
LOG
STAT
STAT
CELL NO. (X 10³)
DAYS IN CULTURE
0
40
80
120
160
200
42
46
50
54
22
26
30
34
24
28
32
36
85

FIGURE 6: Growth curve of primary ten-day cultures. Two sister cultures are represented by the two symbols. For each point a minimum of 400 cells were counted.

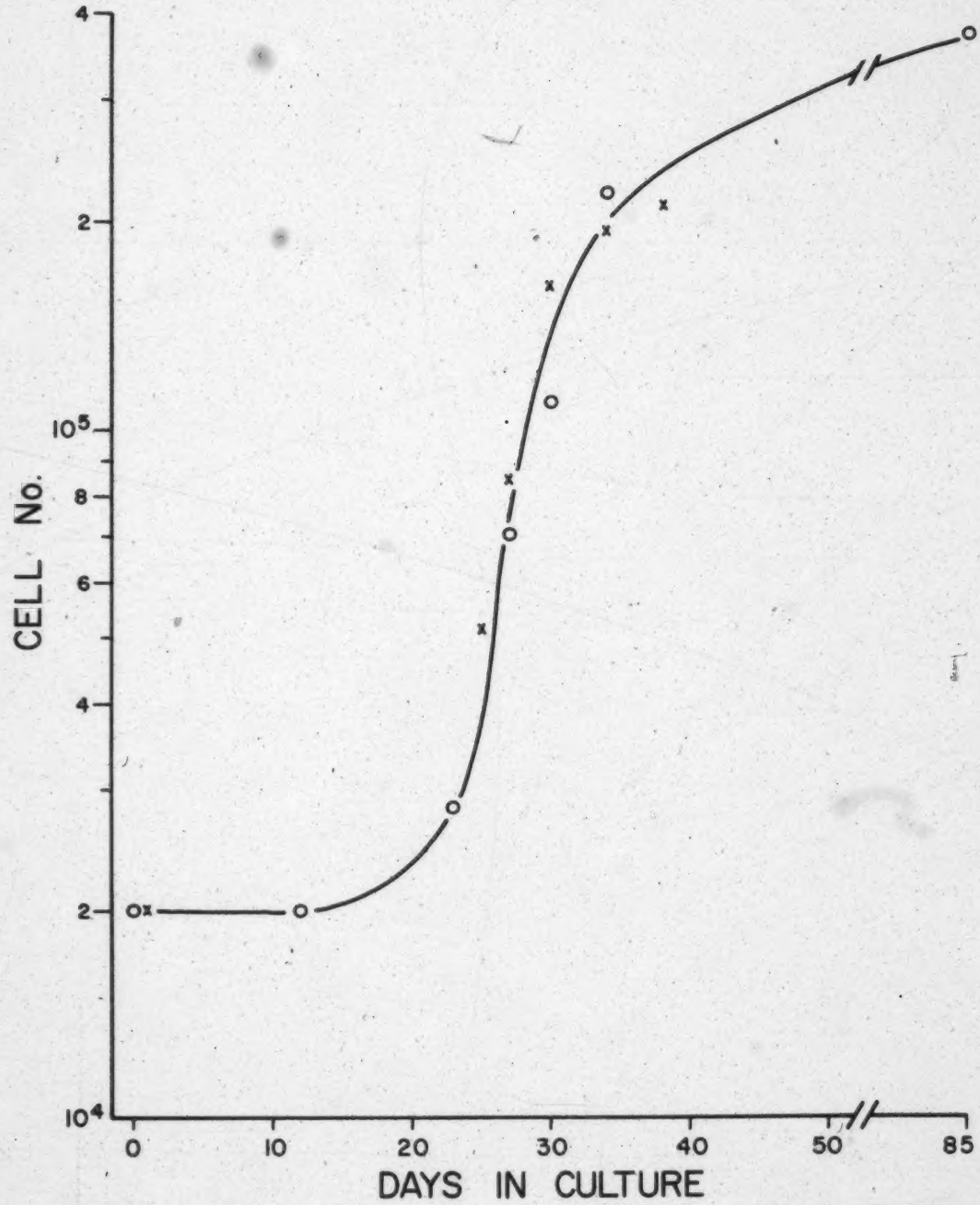
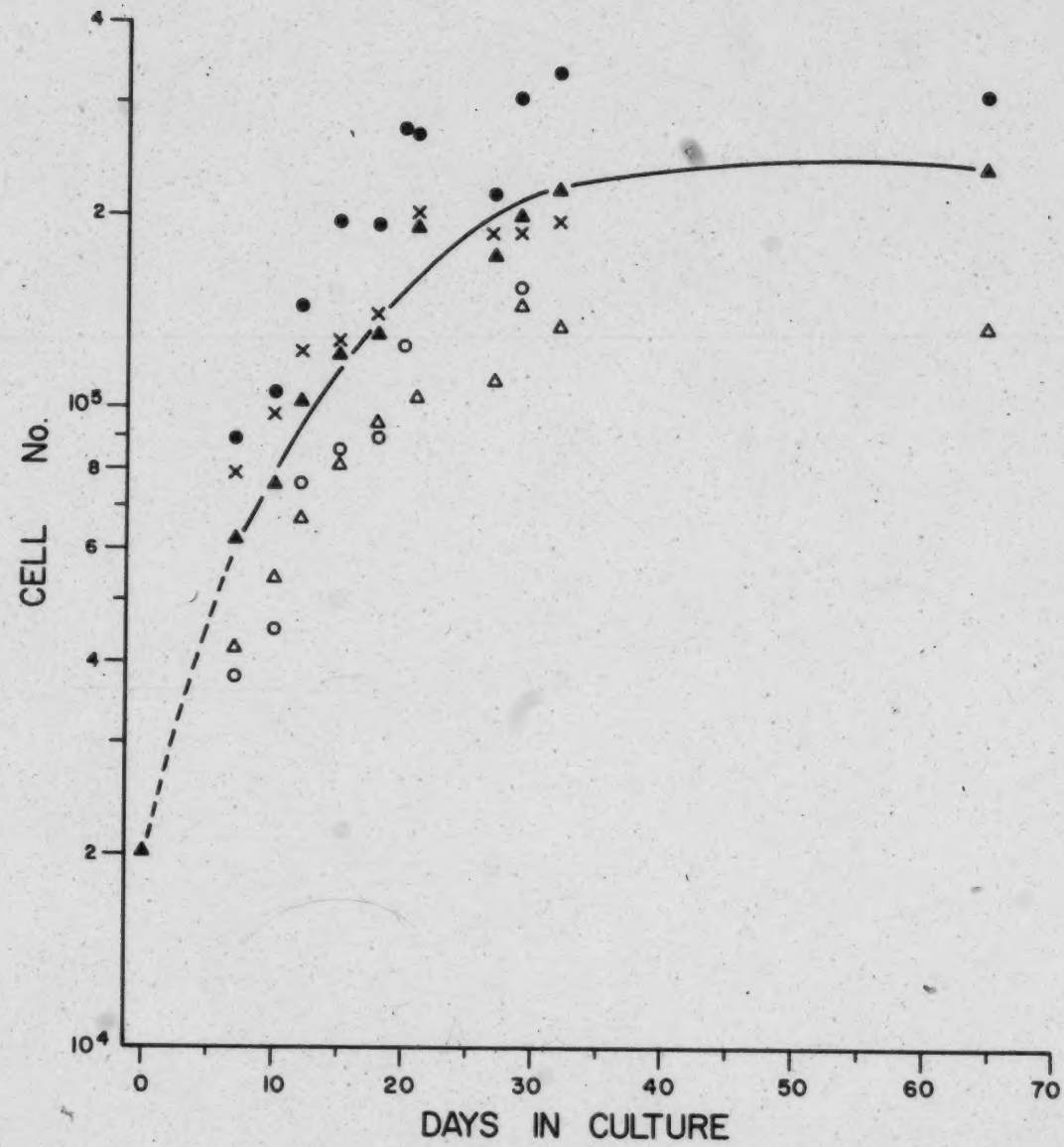

CELL No.
4
2
10⁵
8
6
4
2
10⁴
0
10
20
30
40
50
85
DAYS IN CULTURE

FIGURE 7: Growth curve of primary subcultures. The four replicate subcultures were obtained from the primary normal culture represented in Fig. 2. Shaded triangles represent the mean. A minimum of 400 cells was counted for each point.


CELL No.
4
2
10^5
8
6
4
2
10^4
0
10
20
30
40
50
60
70
DAYS IN CULTURE

Bi- and multinucleate cells comprise about 10% of the population at the end of log phase. In several instances failure to complete cytokinesis was observed to result in viable binucleate cells. These cells are found in both normal and ten-day cultures and primary subcultures (see Figure 9).

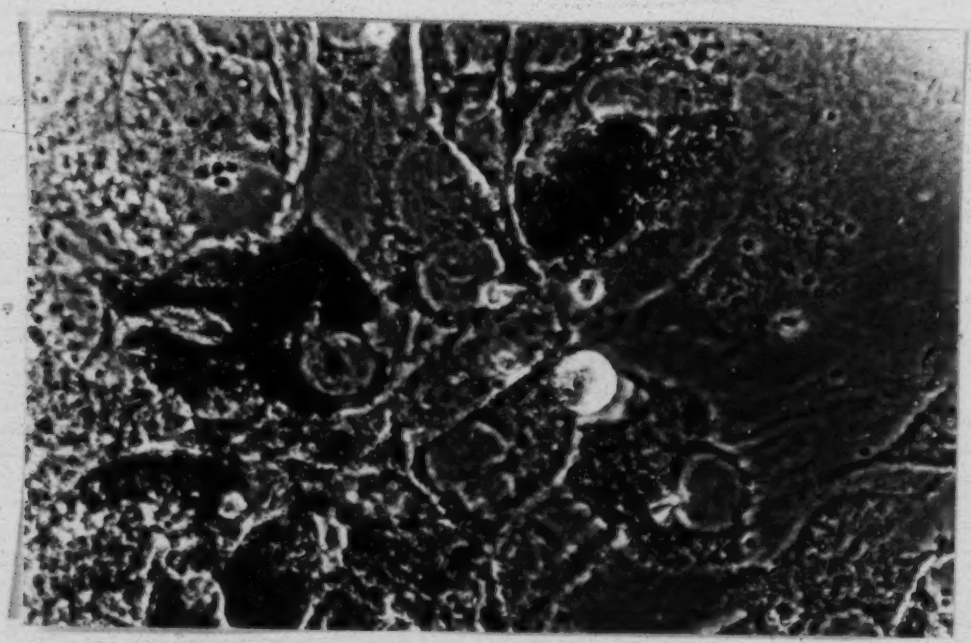
C. Cell Cycle Parameters

The effect of 3.2×10^{-4}M thymidine on the growth rate of two replicate subcultures derived from a normal primary culture is shown in Figure 10. No deviation from the rate of growth immediately prior to the addition of thymidine was detected, and no changes in cell morphology were found. The presence of cytidine, found in mammalian serum (Schneider, 1955), has been shown to protect cells from the inhibitory effect of excess thymidine on DNA synthesis (Morris and Fischer, 1963; Whittle, 1966). Further evidence that inhibition of culture growth is not occurring in the cell cycle experiments described below is found in the mitotic indices from these experiments. If excess thymidine were arresting cells in the DNA synthetic period of the cell cycle, the mitotic index would exhibit very low values until the cells had overcome the block, after which high values would appear. Table I presents the mitotic indices for the normal and ten-day cultures used in the cell cycle experiments. For each value 1000 cells were screened for mitotic figures, including prophase through telophase. The decline and subsequent rise in values predicted in the case of cell cycle arrest is not apparent. It is concluded that culture growth was not arrested and is fairly asynchronous.

In the cell cycle experiments, the synthetic index (S.I.), or percent of the cell population in the DNA

FIGURE 8: Repigmentation in a primary subculture. Subculture was obtained from a primary ten-day monolayer. Magnification X 390.

FIGURE 9: Multinucleate cell in a primary culture. A binucleate and tetranucleate cell can be seen. Culture was in stationary phase. Magnification X 700.



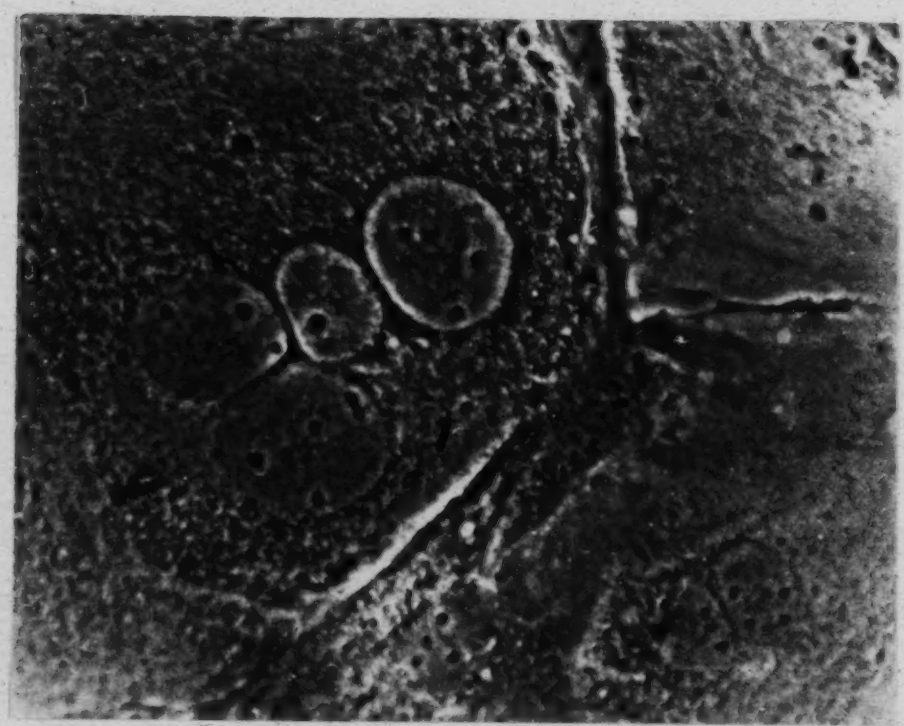


FIGURE 10: Effect of 3.2×10^{-4} M thymidine on the growth of primary subcultures. Arrow indicates time of addition of thymidine (TdR) to medium of two replicate primary subcultures in log phase growth.

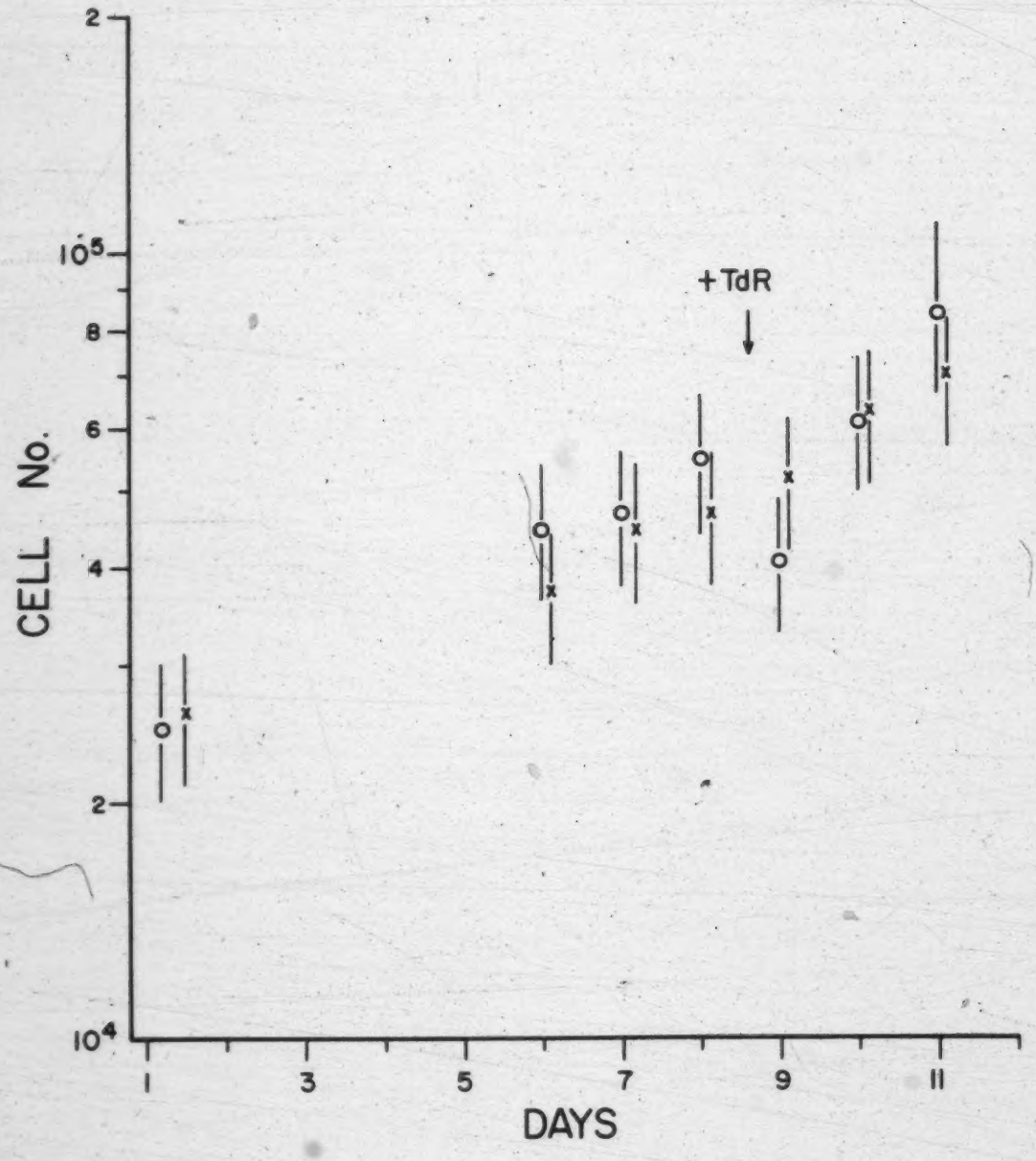

CELL No.
2
10^5
8
6
4
2
10^4
+TdR
1
3
5
7
9
11
DAYS

TABLE I

Mitotic Indices of Normal and Ten-Day Cultures

Hours after incubation in ^{3}H-thymidine	Percent of cells in mitosis	
	normal culture	ten-day culture
4	0.8%	1.7%
8	2.9%	2.3%
10		1.5%
12	1.0%	
16	1.9%	1.6%
22	3.0%	3.1%
28	1.7%	3.9%
34	2.4%	0.7%

synthetic period, was determined for the normal and ten-day cultures by scoring 1000 cell nuclei for the presence of silver grains. Background label was negligible: the mean grain count over 30 labelled nuclei was 130 and over 30 unlabelled nuclei, 4. The S.I. of the first sample, taken 4 hours after incubation in label, was 19% for the normal culture and 22% for the ten-day culture. Later values were slightly lower, indicating maximum incorporation of label into DNA by 4 hours. Therefore 19% and 22% are taken as the S.I.'s for the two cultures.

Howard and Pelc (1953) were the first to divide the cell cycle into four phases: the presynthetic phase or gap (G_1), the DNA synthetic phase (S), the postsynthetic phase (G_2), and mitosis (M). In a cell population the length of each phase can be estimated by measuring the time required for all cells labelled in S to reach mitosis (Quastler and Sherman, 1959). Figures 11 and 12 are the curves obtained for normal and ten-day cultures respectively when percentage of labelled mitoses is plotted against time following incubation in ^{3}H-thymidine. The percentage of labelled mitoses was obtained by scoring 100 cells in metaphase, anaphase or early telophase for the presence of silver grains.

The lengths of the component phase of the cell cycle were calculated from Figures 11 and 12 and are presented in Table II. The interval from 0 hours to the time when 50% labelled mitoses is reached on the ascending side of the first wave approximates the median duration of G_2 & 1/2 M. For both normal and ten-day cultures this is 7 hours. The interval between 50% on the ascending and descending sides of the first wave is taken as an approximation of the median length of S. This value is 36 hours in both cultures. The time required for the completion of one entire cycle, obtained from the inter-

FIGURE 11: Labelled mitoses curve for normal culture. For each point 100 cells in metaphase through telophase were scored for the presence of silver grains. See Text for details of labelling procedure.

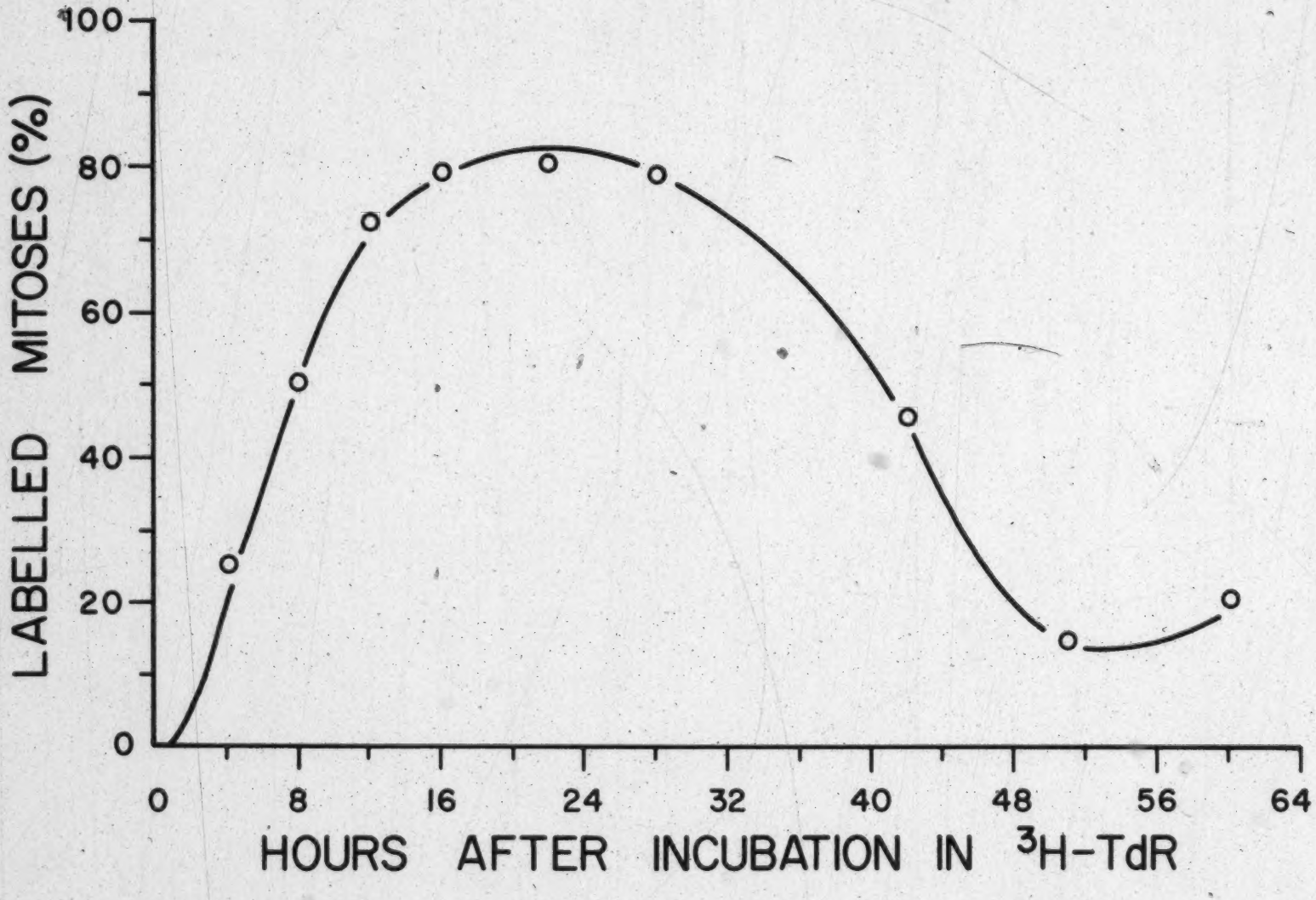

LABELLED MITOSES (%)
100
80
60
40
20
0
0
8
16
24
32
40
48
56
64
HOURS AFTER INCUBATION IN ^{3}H-TdR

FIGURE 12: Labelled mitoses curve for ten-day culture. For each point 100 cells in metaphase through telophase were scored for the presence of silver grains. See Text for details of labelling procedure.

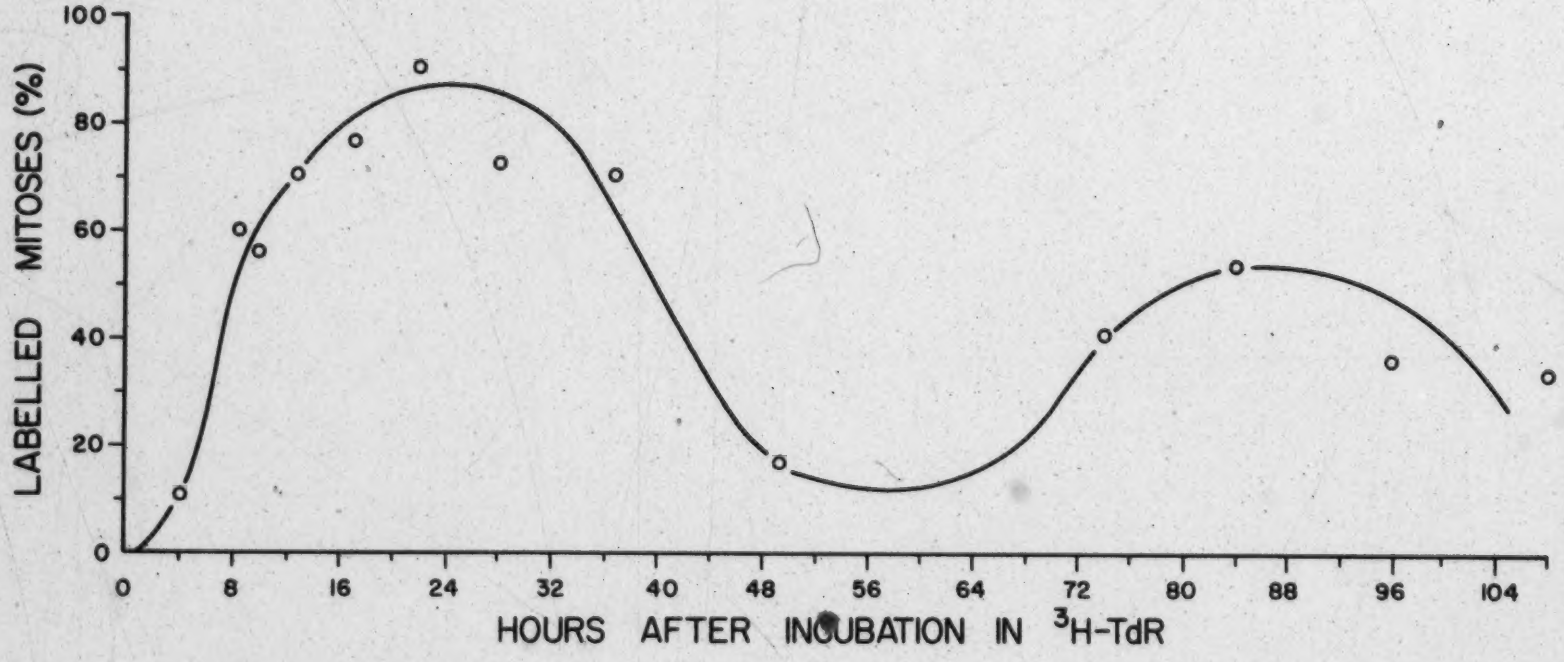

LABELLED MITOSES (%)
100
80
60
40
20
0
0
8
16
24
32
40
48
56
64
72
80
88
96
104
HOURS AFTER INCUBATION IN ^{3}H-TdR

TABLE II

Duration of the Phases of the Cell Cycle
in Normal and Ten-day Cultures

Phase	Normal Culture (hours)	Ten-day Culture (hours)
G_1		25
S	36	36
G_2	6	6
M	2.1	1.8
total		69

val between 50% labelled mitoses on the ascending portions of the first and second waves, is 69 hours in the ten-day culture. The value for G_1 & 1/2 M was obtained by subtraction from total cycle time, and is 26 hours for the ten-day culture. The length of mitosis was measured by observation and represents the time required to proceed from the appearance of condensed chromatin in prophase to the start of cytokinesis. Five cells were timed to obtain the average length of mitosis; for normal cultures this was found to be 2.1 hours and for ten-day cultures, 1.8 hours. By subtraction G_2 is approximately 6 hours in both cultures and G_1 25 hours in the ten-day culture.

From these measurements and the synthetic index, an estimation of the growth fraction, or percentage of cells that are proliferating, can be made for the ten-day culture. According to Lala and Patt (1966),

$$f = (N_S/N)/(N_S/N_G), \qquad (1)$$

where f is the growth fraction, N_S the number of cells in S, N the total number of cells, and N_G the number of cells in the cell cycle. N_S/N is thus the synthetic index, and N_S/N_G can be found from the following equation (Lala and Patt, 1966):

$$N_S/N_G = \exp\left[(G_2+M)(\ln 2)/G\right]\left[\exp\left\{(S/G)(\ln 2)\right\}-1\right] \qquad (2)$$

where G is the total cell cycle time. The growth fraction obtained from equations 1 and 2 is 46%.

Although a second wave of labelled mitoses was not measured for the normal culture, an attempt can be made to estimate the duration of the cell cycle on the basis of several assumptions. In an asynchronous culture, the proportion of cells in each phase of the cycle is directly related to the portion of the total cell cycle time occupied by that phase (Quastler and Sherman, 1959), hence:

$$t_S/S.I. = G \qquad (3)$$

where t_S is the duration of the S phase. However, as Lamerton and Steel (1968) pointed out, this assumes a growth fraction of 1. Modifying Equation 3 to take into account the growth fraction,

$$f(t_S/S.I.) = G \qquad (4)\ ,$$

and using the growth fraction calculated for the ten-day culture, G = 75 hours for the ten-day and 87 hours for the normal culture. This value differs by 6 hours from the value obtained from the labelled mitoses curve for the ten-day culture.

The synthetic index of heavily pigmented cells was also measured in the same experiments. These cells comprised about 2.0% of the population in the normal culture and 3.5% in the ten-day culture. In both cultures the average S.I. for these cells was 1.9%.

DISCUSSION

While the early behavior of iris melanocytes in culture undoubtedly reflects a response to new environmental conditions, much of it bears a resemblance to the behavior of the cells at the pupillary border of the dorsal iris during the early stages of lens regeneration. Depigmentation, for example, is a visible characteristic of the dedifferentiating iris at days 6 to 15 after lentectomy (Dumont and Yamada, 1972). This phenomenon is found in the cultured iris melanocytes within 24-48 hours after plating. In the dorsal iris during regeneration, many cells which will not become part of the lens vesicle undergo partial depigmentation followed by repigmentation. Others do not depigment at all. Whether the cells in culture which remain moderately or heavily pigmented correspond to those in the iris which undergo incomplete or no depigmentation is not known. The presence of heavily pigmented cells in both high and low density areas during the log growth phase suggests that the failure to depigment represents an inherent property of the cell.

Depigmentation of melanocytes in culture has been widely observed (Ebeling, 1924; Doljanski, 1930; Fischer, 1938; Ephrussi and Temin, 1960). Whether a population which does not depigment exists in these cultures has not been reported. Whittaker (1963, 1967) demonstrated three processes responsible for depigmentation in chick retinal pigment cells in vitro: dilution of pigment components by cell growth; decay of the activity of the enzyme tryosinase, the primary enzyme in the melanin biosynthetic pathway; and decrease of tyrosinase synthesis. On the other hand, the process of depigmentation in lens regeneration involves the active extrusion of melanosomes in the presence of macrophages (Eguchi, 1963; Dumont and Yamada, 1972). Accompanying depigmentation in

this system is a steady increase in tyrosinase activity (Achazi and Yamada, 1972). Which of these patterns occurs in depigmenting newt iris cell cultures has not yet been investigated. The presence of macrophages in these cultures for at least a week makes their involvement in the depigmentation process a possibility.

The shedding of fragments of cytoplasm by pigmented cells occurs in both culture and the iris during regeneration. The trails of cytoplasm left on the substrate by migrating melanocytes and the cytoplasmic blebs occasionally seen in depigmenting cells may be the equivalent of 'cytoplasmic shedding' described by Dumont and Yamada (1972) in lens regeneration, whereby melanosomes and cytoplasm are pinched off from the depigmenting cell. These authors found a corresponding decrease in cell volume between days 8 and 15. In normal cultures the ratio of the nuclear-to-cytoplasmic volume of the cells appears to increase by day 5, at the same time that the nucleus changes from kidney-shaped to round or ellipsoid. In regeneration this change of shape is seen at day 7 after lentectomy and is attributed to nuclear swelling (Eguchi, 1963) or reduction of melanosome content (Dumont et.al., 1970). Thus the increase in nuclear-to-cytoplasmic volume in culture may be due to nuclear swelling, loss of cytoplasm, or both.

Prior to mitosis a maximum of 5 or 6 nucleoli appear in the cultured iris cells. This number is also found by electron microscopy beginning at day 2 after lentectomy in cells at the pupillary margin of the dorsal iris; this represents an increase over the 0-3 nucleoli present in cells of the unlentectomized dorsal iris (Dumont et. al., 1970). In the normal cultures the maximum number of nucleoli cannot be seen by phase contrast microscopy until at least a week after plating. Dumont et.al. (1970) observed a fourfold increase in nucleolar size by 4 days

after lentectomy, caused primarily by an increase of the pars granulosa. Increase in nucleolar size is also evident in vitro. The appearance of 5-6 nucleoli per cell in lens regeneration precedes the earliest detectable DNA synthesis by one day and is thought to be the morphological expression of the activation of ribosomal RNA synthesis (Dumont et.al., 1970).

The accelerating effect of trypsin on melanocyte attachment and mitosis is probably due to its effect on the cell surface. Burger (1969) reported that trypsin exposes sites on normal cells which are otherwise found only on malignant cells. Furthermore, Burger (1970) and Rubin (1970) have shown that a lower concentration of trypsin than used in this study will stimulate DNA synthesis in contact-inhibited nonmalignant cells. Burger showed that the site of action of the enzyme in these experiments was restricted to the outer cell surface. Trypsin has also been demonstrated to cause an immediate protein-synthesis-dependent increase in cell volume and mass (Mallucci et.al., 1972). The relation between the action of the enzyme on the cell surface and its stimulatory effects is not yet understood. It is of interest to note that the time required before the onset of mitosis in melanocyte cultures obtained by the trypsin method of dissociation is comparable to the time required by the CMFR-dissociated cells from the ten-day regenerates. The latter have lost some surface components originally present in the normal iris cells (Zalik and Scott, 1972). That a digestive enzyme responsible for this cell surface change might also be activating cellular synthetic activites in regeneration is a possibility.

Mitosis is first observed at day 12 in ten-day cultures and day 21 in normal cultures. Its earlier appearance in the ten-day cultures is not surprising. Ten-day regenerating irises range from Stages II-IV and

contain a portion of cells which have depigmented either partially or completely and have undergone activation of the processes required for DNA synthesis and cell division. The 12-day lag phase seen in ten-day cultures may represent an increasing number of cells entering mitosis between days 12 and 24. Differences among cells in regard to the time required before onset of cell division may reflect their original location in the iris: during regeneration the mitotic index of the pars ciliaris is about one-tenth that of the pars iridica (Yamada and Roesel, 1971). Thus different levels of stimulation may exist among cells of the ten-day cultures. Proximity to other cells in culture may also influence cell division. As mentioned previously, the first cells to divide appear to be in contact with other cells. As Rubin (1966), Takahashi and Okada (1970) and others have demonstrated, cells in culture give off substances which condition the medium, endowing it with growth-stimulating ability. In the early stage of growth in culture these substances might be transmitted from cell to cell. From observation it appears that the number of attached cells in normal cultures prior to the onset of mitosis may be lower than in ten-day cultures. Assuming that the attached cells are the cells synthesizing conditioning substances, this could result in a longer time before the medium is conditioned in normal cultures. Contact between cells would also take longer to be established. Consequently a longer time would elapse before mitosis. The lag phase, which is ten days longer in normal cultures than in ten-day cultures, might also be lengthened for these reasons.

The ten-day cultures have a doubling time of almost half that of the normal cultures. This could be due to a shorter cell cycle time and/or a larger growth fraction in the ten-day cultures. Neither of these possibilites

are ruled out by the present study, although the possibility of a shorter S or G_2 in the ten-day cultures can be excluded. It is possible that previous exposure of irises to the stimulating factors in the lentectomized eye could result in a larger percentage of proliferating cells in ten-day cultures.

The growth rate declines in primary and first subcultures when a cell density of 1.5-2.0 x 10^5 cells per dish is reached. These estimates are of low precision due to the difficulty of counting cells in the clusters arising at late log phase. The highest density found in a monolayer was 3.8 x 10^5, equivalent to a saturation density of 2 x 10^4 cells/cm^2. This is lower than the saturation densities found in mammalian and chick cell monolayers by a factor of 10 or more (see for example Todaro and Green, 1963; Stoker et.al., 1966; Takahashi and Okada, 1970).

In the culture conditions used in this study, heavy pigmentation and cell division appear to be mutually exclusive. The percentage of heavily pigmented cells engaged in DNA synthesis during the log phase (1.9%) is one-tenth that of the total population. This gives a growth fraction of 3.6% for heavily pigmented cells when the t_S/T value for the ten-day culture is used in Equation 4 (Results). Furthermore, as cell proliferation declines, the heavily pigmented population shows an accelerating rate of increase that can only be due to the repigmentation of cells. In many cases a correlation between the synthesis of specialized cell products and absence of cell division has been observed (reviewed by Davidson, 1964); however, a causal relationship has not been established. Recent evidence suggests that culture conditions are responsible for this apparent correlation: condition allowing both cell proliferation and differen-

tiation have been found by Coon (1966), Davies et.al., (1968), Spooner and Hilfer (1971), and others. Cahn and Cahn (1966) found a fraction of embryo extract which allows chick retinal pigment cells to remain pigmented while proliferating.

In the 85-day old monolayer represented in Figures 5C and 6, it can be seen that the ability to redifferentiate has been retained through at least 3 generations by about 50% of the cell population. Whether the nonpigmented fraction at day 85 represents cells which have lost the capacity to differentiate, have simply not reached the stage of differentiation yet, or represents a fast-growing non-melanocyte population remains to be determined.

Bi- and multinucleate cells have been reported to occur in cultures of frog lung cells at about the same frequency as found in the present study. Abnormalities during karyokinesis and cytokinesis were shown by cinematography to give rise to these cells (Burns, 1971).

While the 50% points on the labelled mitoses curve are commonly used for estimating median cell cycle values, a better estimate would be obtained from the points where a change of slope occurs. By inspection of the curves in Figures 11 and 12 it can be seen that such points would be quite close to the 50% points in these experiments. Thus the use of the 50% points seems justifiable.

An estimate of the standard deviation of the values for G_2 can be made as follows. The ascending side of the first wave in Figures 11 and 12 represents the different times spent in G_2 by the population (assuming equal distribution of cells throughout S during labelling and equal rates of passage through S). If the first derivative of the ascending curve shows a normal distribution, the time at which 16% of the mitoses are labelled

will be 1 standard deviation from the time at which the mean (50%) are labelled (Cleaver, 1967). For both cultures this is about 2 hours. However, as Stanners and Till (1960) have shown, the distribution of times in G_2 is not necessarily normal and can vary among cultures of the same kinds of cells.

Calculations of total cell cycle time as in Equation 4 should include the age distribution factor, λ_S, introduced by Lamerton and Steel (1968) to account for the unequal distribution of cells among the cell cycle phases due to logarithmic increase of cell number. Thus

$$G = f(\lambda_S t_S / S.I.). \qquad (5)$$

t_S can be solved for in this equation using the ten-day values and is 0.92. Solving for G of the normal culture, using the (λ_S) and (f) values obtained for the ten-day culture, gives a value of 80 hours for the total cycle time. As mentioned previously, the growth fraction of the normal cultures may well be lower than in the ten-day cultures; this would bring the total cell cycle time closer to that of the ten-day culture.

It is possible, on the other hand, that G_1 is longer in the normal than in the ten-day culture. Among different cell types, both *in vivo* and *in vitro*, the length of the G_1 phase shows much greater variability than any other phase of the cell cycle (Cleaver, 1967). Moreover, within a single cell type, the length of G_1, but not that of other phases, can be affected by a change of environmental conditions such as pH (Sisken and Kinosita, 1961) or serum lot (Tobey et.al., 1967). The factors in the eye-chamber to which the ten-day irises were exposed prior to culture could have been responsible for a shorter G_1 in the ten-day cultures. It is also possible that a shorter G_1 is present at first in ten-day cultures but is lost after a number of generations. That S and G_2 are the same for both cultures is in agreement with results from many

studies of the cell cycle, which show similar durations of S and G_2 throughout many different tissues (Cleaver, 1967).

Little research has been done on the growth of amphibian cells *in vitro*, partly because their nutritional requirements are poorly defined in comparison to those of chick and mammalian cells. At 25°C., the optimal temperature for growth, these cells grow more slowly than mammalian cells. Primary cultures of liver cells of *T. cristatus* grown in modified Eagle's medium are reported to have an S period of 24-48 hours (Mitchison, 1971; Callan, 1972). Adult frog kidney cells grown in modified Eagle's have a cell cycle time of 46 hours, with a $G_1/S/G_2$ of 16/22.3/7.7 hours (Malamud, 1967).

In studies of the cell cycle in the dedifferentiating dorsal iris, the values for the duration of S were found to be between a minimum of 19 and maximum of 33 hours and total cell cycle time between 65 and 80 hours (Zalik and Yamada, 1967). Under the more defined labelling conditions of the present study a value of 36 hours for S and 69 hours for total cycle time was obtained using cells from 10 -day regenerates. These values are close to those reported by these authors for 15-day regenerates. Thus it appears that, at least with respect to cell cycle parameters, cultured iris cells do not differ significantly from those in the activated iris. These findings suggest that a meaningful study of factors controlling lens regeneration may be possible.

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